Original Article
Effects of 18α-glycyrrhizin on TGF-β1/Smad signaling pathway in rats with carbon tetrachloride-induced liver fibrosis

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Abstract: Background: Glycyrrhizin has various pharmacological effects including hepato-protection. This study aimed to investigate the potential mechanism underlying the protective effects of 18α-glycyrrhizin (18α-GL) in rats with carbon tetrachloride (CCl4) induced liver fibrosis. Methods: Male Sprague-Dawley (SD) rats were randomly divided into control group, fibrosis group, 25 mg/kg 18α-GL group and 12.5 mg/kg 18α-GL group. Rats in experimental groups were subcutaneously injected with 40% CCl4 twice weekly for 8 weeks. Immunohistochemical examination was carried out to detect the protein expressions of collagen I, collagen III, TGF-β1, p-Smad2, p-Smad3, Smad 7 and SP-1, in the liver, and the mRNA and protein expressions of these genes were determined in the liver by real time PCR and Western blot assay, respectively. Results: 18α-GL ameliorated histological changes and significantly suppressed collagen deposition. 18α-GL significantly decreased the mRNA expressions of TGF-β1, Smad2, Smad3 and SP-1 in the liver. Immunohistochemical staining revealed that TGF-β1, p-Smad2, p-Smad3 and SP-1 expressions reduced following 18α-GL therapy. Western blot assay showed p-Smad2, p-Smad3, smad2 and smad3 expressions decreased after 18α-GL treatment. The mRNA and protein expression of Smad7 remained unchanged. Conclusion: 18α-GL is able to attenuate CCl4 induced liver fibrosis in rat.

Keywords: 18α-glycyrrhizin, liver fibrosis, collagen, TGF-β1, Smad

Introduction

Liver fibrosis and cirrhosis arise from chronic liver injury caused by a variety of etiological factors including viruses, alcohol, metabolic syndrome and autoimmune diseases [1]. Understanding the molecular mechanisms that regulate the hepatic inflammatory and fibrotic processes is crucial for the development of therapeutic interventions designed to tackle this disabling and fatal condition. Fibrosis is a progressive pathological process in which liver myofibroblasts respond to injury by promoting the replacement of normal hepatic tissues with scar-like matrices composed of cross-linked collagen [2]. The extracellular matrix (ECM) is increasingly recognized as an important mediator of cellular survival and proliferation [3]. Multiple cytokines participate in the process of liver fibrosis in autocrine and paracrine dependent manners [4].

To date, effective treatments for liver fibrosis and cirrhosis have not been developed yet, and liver transplantation is the primary choice of treatment. Thus, it is imperative to develop effective anti-fibrotic agents. Great progresses have been made in the pharmacotherapy of liver fibrosis. Recent surveys in the United States showed that vitamins and herbal supplements could serve as alternative therapies for chronic liver diseases [5], indicating that active ingredients extracted from plants have a great potential for the treatment of liver diseases [6]. Some herbal medicines have been found to effectively improve the liver fibrosis, with moderate to low toxicity. A variety of botanical ingredients have been tested in in vitro, in vivo and in patients [7-9]. Glycyrrhizin (GL), a triterpenoid saponin glycoside, is a sweet-flavored component of licorice root, and has broad pharmacological activities. GL is an inhibitor of hepatitis B virus and hepatitis C virus, and can improve the
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Liver function [10-12]. A 26-week, randomized, phase II trial showed that GL infusion could reduce alanine transaminase levels in chronic hepatitis C [13]. GL has 2 isomers: 18α-GL and 18β-GL. Due to the safety and efficacy of 18α-GL, we investigated the ability of 18α-GL to protect against carbon tetrachloride (CCl₄)-induced liver fibrosis in rats, and the impact of 18α-GL on fibrogenesis. Our results showed that 18α-GL improved liver fibrosis by acting on the TGF-β/Smad signal pathway, suggesting that 18α-GL possesses anti-fibrogenic properties.

Materials and methods

Animals and grouping

Male Sprague-Dawley (SD) rats weighing 180-200 g were purchased from the Animal Center of School of Medicine, Shanghai Jiao Tong University. Animals were randomly divided into four groups: control group (n=8), liver fibrosis group (n=12), 12.5 mg/kg 18α-GL group (n=12) and 25 mg/kg 18α-GL group (n=12). In later three groups, fibrosis was induced by subcutaneous injection of 0.2 ml/100 g CCl₄ in olive oil twice weekly for 8 consecutive weeks (the first dose was doubled). In control group, olive oil was subcutaneously injected. In liver fibrosis group, animals were intraperitoneally treated with normal saline (NS). In 12.5 mg/kg 18α-GL group and 25 mg/kg 18α-GL group, animals were intraperitoneally treated with 18α-GL at 25 and 12.5 mg/kg, respectively, once daily for 8 weeks. 18α-GL (Chia-tai Tianqing Pharmaceutical Co., China) and all other chemicals and reagents were of analytical grade. All rats were anesthetized after 8 weeks, and the livers were collected. Liver tissues were fixed in 10% formaldehyde for 24 h and stored at -80°C. The animal procedures were conducted according to the institutional and national guidelines for the Care and Use of Laboratory Animals, and the whole study was approved by the Ethics Committee of China Shanghai Jiao Tong University.

Histological examination

Liver tissues were embedded in paraffin, and processed for hematoxylin-eosin (H&E) and Masson’s trichrome staining, and pathological examination and scored by two pathologists blind to this study. Four fields were randomly selected from each section and histopathological evaluation was performed twice.

RNA isolation and real time PCR

Total RNA was extracted from the liver tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and subjected to RT using PrimeScript® RT reagent Kit (TAKARA, DRR037S, Japan). Real time PCR was performed according to the manufacturer’s instructions using SYBR® Premix Ex Taq™ Kit (TAKARA., DRR041A, Japan) on the ABI-Prism 7700. Each experiment was performed in triplicate. GAPDH was used as an internal control. The primers are listed in Table 1. The mRNA expression of target genes was normalized to that of GAPDH according to previously reported [14].

Immunohistochemical analysis

5-μm sections were de-paraffinized and incubated in phosphate PBS containing 3% H₂O₂ for 10 min to block the endogenous peroxidase activity. Antigen retrieval was carried out in 0.01 M citric acid buffer. Trypsin treatment was done for collagen I and III in pH 6.0 sodium citrate buffer, high pressure treatment for p-Smad2, and microwave treatment for other antibodies. Sections were then rinsed thrice with PBS and blocked with Power Block™ Universal Blocking reagent (Biogenex, HK085-5KE, USA) for 10 min, followed by incubated overnight with following primary antibodies: collagen I ([1:400], Abcam); collagen III ([1:500],

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<th>Gene</th>
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<tr>
<td></td>
<td>R: 5'-GGAGGAGGTTGCACTC-3’</td>
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<td>TGF-β1</td>
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<tr>
<td></td>
<td>R: 5’-TACCTGACACAGCATCACC-3’</td>
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</table>
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Abcam); TGF-β1 ([1:60], Abcam); p-Smad2 ([1:100], NovusBio); p-Smad3 ([1:100], Millipore); Smad7 ([1:200], R&D); Sp1 ([1:80], Abcam). Then, a 30-min incubation with corresponding secondary antibodies was performed using the Super Sensitive™ Polymer-HRP Two-step Histostaining Reagent (Biogenex, HK5-18/9-YAK, USA). Sections were visualized with Biogenex stable 3,3’-diaminobenzidine tetrahydrochloride (DAB). As a negative control, the primary antibody was replaced with PBS. Sections were counterstained, mounted, and examined by microscopy. Positive cells had brown-yellow granules. Five fields were randomly selected from each section, and the color image analysis system (Image-ProPlus (IPP) 6.0 software) was used to estimate the protein expression.

Measurement of hepatic hydroxyproline content

Total hepatic hydroxyproline (HYP) levels were determined in the hydrolysates of liver samples as described previously [15].

Western blot assay

Liver tissues were lysed in 200-ml RIPA buffer (0.05 M Tris-HCl [pH 7.4], 0.15 M NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin and 10 mg/ml leupeptin). Protein concentrations were measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Proteins were subjected to SDS-PAGE and transferred onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The blots were probed with the following primary antibodies: Smad2/phospho-Smad2 (pSmad2), Smad3/phospho-Smad3 (pSmad3), actin (Cell Signaling Technology). The concentration of primary antibody was from the manufacturer’s instructions. Detection was done with enhanced chemiluminescence (Invitrogen, Carlsbad, CA, USA). Bands were scanned and analyzed using NIH Image J (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Statistical analysis

Statistical analysis was performed with SPSS version 11.0 statistic software package. Data
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**Results**

**Histopathological examination and detection of liver collagen deposition**

The effects of 18α-GL on the CCl₄-induced liver fibrosis were evaluated by histopathological examination of the liver by H&E staining (Figure 1). In control group, normal structure and no pathologic changes were present, and 18α-GL succeeded in inducing liver fibrosis.

On the one hand, HYP content can display the content of collagen in the liver, and on the other hand, the content of HYP in the liver can directly reflect the degree of hepatic fibrosis. The HYP content of animals in 25 mg/kg and 12.5 mg/kg 18α-GL was considered statistically significant.

Comparisons between groups were performed with analysis of variance (ANOVA), Student's t-test or Kruskal-Wallis test. A value of $P<0.05$ was considered statistically significant.

**Figure 3.** Effects of 18α-GL on the collagen I and III expression in Rat Liver (immunohistochemistry). Collagen I (A-D) and collagen III (E-H) expression of control group (A, E), liver fibrosis group (B, F), 12.5 mg/kg 18α-GL group (C, G) and 25 mg/kg 18α-GL group (D, H). Positive cells had brown granules (100×). (I, J) Ratio of collagen I and III expression. Data are expressed as means ± standard deviation *$P<0.05$ vs liver fibrosis group.

**Figure 4.** Effects of 18α-GL on the TGF/Smad mRNA expression. qPCR was performed to detect the mRNA expression of TGF-β1, Smad2/3/7 and SP-1. Data are expressed as means ± standard deviation *$P<0.05$ vs liver fibrosis group.
GL groups (167.00±55.31 μg/g, 184.25±49.33 μg/g) were significantly lower than that in liver fibrosis group (273.38±66.22 μg/g, \( P < 0.05 \), Figure 2).

Liver collagen accumulation was determined by immunohistochemistry of liver tissues (Figure 3). In control group, little collagen was present. In liver fibrosis group, collagen I and III were found around the portal vein, in the central venous and sinusoidal area, and the intervals along the fibers showed diffuse distribution. However, in 18α-GL group, collagen fibers mainly located in the central lobular and portal areas. 18α-GL significantly reduced the collagen deposition and distribution.

**Effects of 18α-GL on the expression of TGF-β1/Smads**

In control group, TGF-β1 protein expression was only detectable in cells surrounding the portal vein and central veins of hepatic lobules with a small amount of TGF-β1 surrounding cells. In liver fibrosis group, TGF-β1 was over-expressed, and mainly distributed in cells sur-
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rounding the portal area, sinusoidal endothelial cells, some hepatocytes and fiber septum. The TGF-β1 mRNA expression reduced markedly (Figure 4). The TGF-β1 positive cells reduced significantly in 18α-GL groups when compared with liver fibrosis group (Figure 5; Table 2).

Smad3 and Smad2 are key downstream effectors of TGF-β signaling pathway [16, 17] and p-Smad2 is the activated form of Smad2. Our results showed it was not expressed in liver tissues under normal conditions, but its expression increased following CCl₄ treatment. p-Smad2 expression increased in fibroblasts and perisinusoidal and inflammatory cells in the proliferative fibrous tissues surrounding the lobes, but p-Smad2 and smad2 reduced in 18α-GL groups (Figures 5, 6; Table 2). In addition, cytoplasm-positive cells were also observed in 18α-GL groups, suggesting that 18α-GL also plays a role in preventing its nuclear translocation. p-Smad3 is the activated form of Smad3 and not expressed in the liver under normal conditions. Hepatic fibrosis elevated the expression of Smad3 in stromal cells. In 18α-GL group, p-Smad3 and smad3 expression significantly decreased (Figures 5, 6; Table 2).

In control group, little Smad7 was detected, but a small number of Smad7 positive cells were detectable following CCl₄ treatment in the fibrous septa and portal area. In rats receiving 18α-GL treatment, Smad7 protein and mRNA expressions were equivalent to those observed in the control group (Figure 5; Table 2).

There is evidence showing that Sp-1 and Smad proteins cooperate to induce the expression of alpha 2 (I) collagen in human glomerular mesangial cells following TGF-β1 treatment [18]. HCV-induced transcription factors AP-1, Sp-1, NF-κB and STAT-3 are involved in TGF-β1 gene expression [19].

In the normal liver tissues, no SP-1 expression was observed, but over-expression of SP-1 was noted not only in the fiber septum but in the hepatocyte nuclei and some duct cells in the presence of liver fibrosis (Figure 5; Table 2). SP-1 expression reduced significantly in the fiber septum in rats receiving 18α-GL treatment.

Discussion

TGF-β1 has been identified as the most profibrotic cytokine, and can elevate the expression of collagen I in hepatic stellate cell (HSC), promote their transition to a myofibroblast-like phenotype, and modulate key elements of ECM in the homeostasis [20, 21]. Numerous studies have revealed TGF-β1 elevated in tissues and organ lesions, particularly in case of fibrosis. TGF-β1 is closely related to the progression of liver fibrosis. Marek et al. found both serum TGF-β1 concentration and TGF-β1 mRNA expression in the liver were useful prognostic markers in patients with hepatitis C undergoing antiviral therapy [22]. Castilla et al. found that TGF-β1 mRNA expression correlated closely with the mRNA expression of procollagen Type I (r=0.94), serum procollagen Type III peptide (r=0.89) and histologic activity index (r=0.73) [23]. All patients with increased fibrogenic activity had increased levels of TGF-β1 mRNA. Following liver injury, activated TGF-β ligands are detectable in the liver and may induce downstream signals in all cell types investigated.

<table>
<thead>
<tr>
<th></th>
<th>TGFβ1</th>
<th>p-Smad2</th>
<th>p-Smad3</th>
<th>Smad7</th>
<th>SP-1</th>
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<td>Control group</td>
<td>0.0560±0.0134</td>
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<td>0</td>
<td>0.0068±0.0032</td>
<td>0</td>
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<td>Liver fibrosis group</td>
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<td>0.0866±0.0136</td>
<td>0.0500±0.0119</td>
<td>0.0176±0.0034</td>
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<td>12.5 mg/kg 18α-GL group</td>
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<td>0.0346±0.0120*</td>
<td>0.0216±0.0064*</td>
<td>0.0192±0.0043</td>
<td>0.1790±0.0596*</td>
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<td>25 mg/kg 18α-GL group</td>
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<td>0.0336±0.0062*</td>
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<td>0.0164±0.0067</td>
<td>0.1430±0.0597*</td>
</tr>
</tbody>
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Footnotes: *P<0.05 vs. liver fibrosis group; †P<0.05 vs. to 25 mg/kg 18α-GL group.

Figure 5. Western blot assay of p-Smad2 and p-Smad3 expression in the rat liver after 18α-GL treatment.

Figure 6. Western blot assay of Smad2/3 and p-smad2/3 expression in the rat liver after 18α-GL treatment.
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Several experiments in which TGF-β expression is dis-regulated in the liver by adenovirus or in transgenic mice have revealed the importance of TGF-β in the HSC activation and fibrogenesis [24]. TGF-β signaling pathway is required for the α-SMA organization and stress-fiber formation, while myofibroblasts are fully stimulated via autocrine TGF-β signaling pathway, display strong intrinsic R-Smad activation and, importantly, lack Smad7 up-regulation [25, 26]. Such anti-TGF-β approaches have been established and successfully used for the treatment of experimental fibrosis. Dominant-negative or soluble TβRlIs have been applied to suppress fibrosis in mice and rats upon dimethyl nitrosamine-, CCl₄- or bile duct ligation-induced liver injury [27]. Experimental TGF-β1 directed antibodies, small molecule inhibitors and RNA interference have also been shown to suppress fibrosis [28]. Although many of these approaches have shown promising results in animal models for more than a decade, there is currently still no effective treatment available for the therapy of human diseases. Based on the in vitro studies of rat and mouse HSCs and in vivo animal models of liver injury, several conclusive statements about liver fibrosis can be made: TGF-β is required for the liver fibrosis, and reduction of TGF-β is helpful to inhibit fibrogenesis [27]. In the present study, elevated TGF-β1 expression was found in rats with CCl₄-induced liver fibrosis, which was attenuated by intraperitoneal 18α-GL. We speculate that 18α-GL can inhibit TGF-β1 production, thereby suppressing the TGF-β1 signal cascade in HSCs and thus inhibiting hepatic fibrosis.

TGF-β1 exerts its biological properties by binding to high-affinity receptors with intrinsic serine/threonine kinase activity and subsequently activate intracellular effectors known as Smads. Upon TGF-β1 binding to the TGF-β type II receptor, the type II receptor kinase phosphorylates the GS domain of TGF-β type I receptor, leading to the activation of type I receptor [29]. Activated type I receptors then trigger the downstream Smad signaling pathway by phosphorylating Smad2 and Smad3 at two serine residues in the SSXS motif of their C terminal [30]. Phosphorylated Smad2 and Smad3 form oligomeric complexes with Smad4, which then translocate into the nucleus and mediate the transcriptional regulation of target genes. Thus, Smad proteins transmit signals directly from the receptor kinase to the nucleus [31]. On the other hand, Smad7 antagonizes this signaling pathway by inhibiting the phosphorylation of R-Smads, forming a negative feedback loop to control TGF-β1 responses [32]. TGF-β mediates the activation of Smad2 primarily in undifferentiated cells and Smad3 primarily in trans-differentiated cells [33]. Maximal expression of collagen type I in the activated HSCs requires Smad3 in Smad3 heterozygous and Smad3 homozygous knockout mice treated with a single intragastric dose of CCl₄ [34]. Liu et al. also proposed that suppressing TGF-β-induced Smad2/Smad3 phosphorylation and nuclear translocation in HSCs was helpful to attenuate fibrosis [35]. Application of Smad2/Smad3 antisense oligonucleotides or cDNA is also effective to block or inhibit the biological functions of TGF-β [28]. IFN-γ induces anti-fibrotic effects in hepatocytes via phosphorylating STAT-1, up-regulating Smad7 expression and impairing TGF-β signaling pathway [36]. Smad7 acts to inhibit the phosphorylation of Smad2 and Smad3, nuclear translocation of activated Smad complexes, and activation of (CAGA) (9)-MLP-Luc, resulting in decreased collagen I expression and complete inhibition of TGF-β signal transduction [25, 37]. Hepatocyte-specific Smad7 expression attenuates TGF-β-mediated fibrogenesis and protects against liver injury [38]. R-Smad and I-Smad expression imbalance is one of the molecular mechanisms of liver fibrosis. Theoretically, gene therapy is able to increase Smad7 expression or decrease Smad2/Smad3 expression.

In order to further elucidate the mechanism by which 18α-GL protects against liver fibrosis, the impact of 18α-GL on the downstream transcription factors p-Smad2/3 and Smad7 was further investigated. 18α-GL significantly inhibited p-smad2 and p-smad3 expression, highlighting a potential anti-fibrotic mechanism. Smad3 protein expression was persistently high in the whole processes of hepatic fibrosis, while Smad7 protein expression only transiently increased in the early stage of fibrosis and low expression was observed in the middle and late stages. In addition, only low Smad7 expression which was not significantly altered in the group receiving 18α-GL. GL treatment may be due to up-regulated Smurf2 inducing Smad7 degradation [39].

SP-1 is an important transcription factor involved in almost all cellular functions including proliferation, apoptosis and differentiation. Local hypoxia and free radicals in tumors can
induce SP-1 over-expression [40]. In the study on nerve cells, oxidative stress-induced SP-1 up-regulation has been found to be an adaptive response to cell damage [41]. We postulate that over-expression of SP-1 in the hepatocytes in case of liver fibrosis is ascribed to the oxidative stress.

SP-1 is also involved in the regulation of type I collagen expression. SP-1 may be involved in the activation of HSC collagen I gene promoter regions FP1 and FP2. Over-expression of SP-1 enhances the promoter activity, and thereby promotes collagen I expression, while the FP1 and FP2 variations reduce the promoter activity [42]. Zhang et al. found that, in the absence of SP-1, Smad3/Smad4 failed to promote collagen I α-2 (COL1A2) transcription. Only in the presence of SP-1 could Smad3/Smad4 promote COL1A2 transcription [43]. COL1A1 gene transcription has also been reported to be regulated by SP-1 [44]. Our results showed that in liver fibrosis group, SP-1 was over-expressed in HSCs, accompanied by Smads and collagen I over-expression, illustrating a synergistic role in the Smad signaling pathway. Our previous in vitro studies also revealed that an active metabolite of 18α-GL reduced SP-1 DNA binding activity in HSC cell lines [45]. In addition, in vivo 18α-GL reduced SP-1 expression, suggesting a mechanism by which 18α-GL reduces collagen secretion of HSCs to attenuate liver fibrosis.

In summary, 18α-GL effectively ameliorates the CCl₄-induced liver fibrosis in rats by, markedly inhibiting collagen I and III expression and altering TGF-β1/Smad signaling pathway. In addition, 18α-GL also inhibits TGF-β1, p-Smad2/smad2, p-Smad3/smad3 and SP-1 expression. Therefore, 18α-GL may impede the signal transduction of TGF-β1 and inhibit the pro-fibrotic effect of TGF-β1 at multiple levels. In conclusion, 18α-GL may act on multiple pathways to improve collagen expression and suppress liver fibrosis.

Acknowledgements

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Disclosure of conflict of interest

None.

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